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Cloning and nitrate induction of nitrate reductase mRNA

(barley/gel separation/hybrid-selected translation)

CHI-LIEN CHENG*, JULIA DEWDNEY*, ANDRIS KLEINHOFS†, AND HOWARD M. GOODMAN*‡

*Department of Molecular Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, MA 02114; †Department of Agronomy and Soils, and Program in Genetics and Cell Biology, Washington State University, Pullman, WA 99164

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ABSTRACT **Nitrate is the major source of nitrogen taken from the soil by higher plants but requires reduction to ammonia prior to incorporation into amino acids. The first enzyme in the reducing pathway is a nitrate-inducible enzyme, nitrate reductase (EC 1.6.6.1). A specific polyclonal antiserum raised against purified barley nitrate reductase has been used to immunoprecipitate *in vivo* labeled protein and *in vitro* translation products, demonstrating that nitrate induction increases nitrate reductase protein and translatable mRNA. A partial cDNA clone for barley nitrate reductase has been isolated and identified by hybrid-selected translation. RNA blot-hybridization analysis shows that nitrate induction also causes a marked increase in the steady-state level of nitrate reductase mRNA.**

The nitrate reducing pathway is the most important route for higher plants, algae, and a variety of bacteria to assimilate nitrogen from their environment. Nitrate is reduced to nitrite and nitrite to ammonia, the end product of nitrate reduction, which is then incorporated into amino acids. Therefore, nitrate is ultimately the major nitrogen source for essentially all living organisms. Higher plants alone assimilate 2×10^4 megatons (1 metric ton = 1000 kg) of nitrogen each year through this pathway. Nitrate reductase (EC 1.6.6.1), the first enzyme in the nitrate reducing pathway, is thought to play a critical role. Nitrate reductase levels in higher plants have been shown to fluctuate in response to changes of environmental conditions such as light, temperature, pH, CO_2 and oxygen tensions, water potential, nitrogen source, and other factors—changes that usually also influence the capacity of the organisms to assimilate nitrate (1).

Despite numerous correlations of these environmental factors to nitrate reductase activity, the level(s) at which the regulation takes place is far from clear. The best-studied factor that regulates nitrate reductase activity is its own substrate, nitrate. In 1957, Tang and Wu (2) demonstrated that in rice seedlings nitrate reductase activity is induced in response to nitrate. Since then, the phenomenon has been widely observed in many plant species (3–5). Using cultured tobacco cells, Zielke and Filner (6) showed that nitrate reductase activity induced by nitrate is an activity of a protein synthesized *de novo* after addition of inducer. The definitive proof that nitrate reductase is regulated by enzyme synthesis and/or degradation rather than by an activation-inactivation mechanism comes from the study of Sommers *et al.* (7) in barley. Higher plant nitrate reductase is a pyridine nucleotide-dependent enzyme that has been purified from several species (8–11). The enzyme appears to be a homodimer with a subunit M_r range of 110,000–140,000 in different species. By using antiserum made against purified barley nitrate reductase, Sommers *et al.* (7) showed a positive correlation between nitrate reductase activity and an increase in the M_r 110,000 immunologically cross-reacting band on electrophoretic transfer blots.

We have used the specific polyclonal antiserum against barley nitrate reductase to immunoprecipitate *in vivo* labeled protein and *in vitro* translation products, demonstrating that nitrate induction increases translatable mRNA and that this increase correlates to the induction of nitrate reductase protein. We have also isolated cDNA clones for barley nitrate reductase and by RNA blot-hybridization analysis have shown that nitrate induction causes a marked increase in the steady-state level of nitrate reductase mRNA.

MATERIALS AND METHODS

Growth and *in Vivo* Labeling of Plants. *Hordeum vulgare* L., cv. Steptoe (barley) seedlings were grown in vermiculite at 18°C for 5 days with constant illumination [300 microeinsteins/m^2 per s (1 einstein = 1 mol of photons)] and watered daily with deionized water. Seedlings were then irrigated twice at 12-hr intervals with a nitrate-containing solution for induction or nitrate-free solution for controls (7). Ten 5-day-old seedlings were excised and fed with 250 μl of the above solutions containing 100 μCi of [^{35}S]methionine (New England Nuclear, 1000 Ci mmol/liter; 1 Ci = 37 GBq) for 12 hr. Excised leaves were frozen immediately in liquid nitrogen and stored at −80°C.

Protein and RNA Extractions. Proteins were extracted as described (7). RNA was extracted from the frozen leaves by the guanidinium/phenol method (12), except that the extraction procedure was carried out at 4°C. Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography (13).

***In Vitro* Translation and Immunoprecipitation.** *In vitro* translation conditions were as described by the suppliers (New England Nuclear or Bethesda Research Laboratories). The crude protein from one plant equivalent or 25 μl of *in vitro* translation mixture was allowed to react with nitrate reductase antibody-coated protein A-Sepharose beads (14) at 4°C for 2 hr in 1 ml of buffer containing 25 mM Tris·HCl (pH 7.6), 350 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% $NaDodSO_4$. The beads were washed with the same buffer three times at 4°C, resuspended in $NaDodSO_4$/gel loading buffer, boiled for 2 min, and then subjected to gel electrophoresis. After drying of the gel and radioautography, the region of the gel corresponding to the position of nitrate reductase was excised and the radioactivity was determined by scintillation counting. This value divided by the total trichloroacetic acid-precipitable radioactivity of the *in vitro* translation (prior to immunoprecipitation and electrophoresis) was used to estimate the relative abundance of nitrate reductase mRNA.

RNA Size Fractionation and Construction of cDNA Libraries. Total poly(A)$^+$ RNA was fractionated on a native sucrose gradient as described by Cashmore (15). Two micrograms of

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Abbreviations: kb, kilobase(s); bp, base pair(s).

‡ To whom reprint requests should be addressed.

the sucrose gradient-fractionated poly(A)$^+$ was used to construct a cDNA library in an expression vector. Double-stranded cDNA was synthesized according to Gubler and Hoffman (16) and, after *Eco*RI linker addition, was cloned into bacteriophage λgt11 (17). Antiserum was diluted 1:1000 to screen for the recombinant phages expressing nitrate reductase protein according to the procedure described in ref. 17. Twenty micrograms of sucrose gradient-fractionated RNA enriched for nitrate reductase RNA was subjected to methylmercuric hydroxide/agarose gel electrophoresis. The conditions were as described in Maniatis *et al.* (18). One percent low-melting agarose was used. RNA was extracted from each 2-mm gel slice and 1/10th of the RNA was used for *in vitro* translation and immunoprecipitation analysis. The RNA population containing the highest nitrate reductase mRNA activity was used in constructing a (dG–dC)-tailed cDNA library in the plasmid vector pUC12 (19).

Hybrid Selection. Hybrid selection conditions were essentially as described by Miller *et al.* (20). Hybridization was in 50% formamide/10 mM Pipes, pH 6.4/0.4 M NaCl/450 μg of poly(A)$^+$ RNA per ml at 37°C for 12 hr. Filters were then washed five times with 0.3 M NaCl/30 mM sodium citrate/0.5% $NaDodSO_4$, five times with 30 mM NaCl/3 mM sodium citrate, and two times with 2 mM EDTA, at 70°C. The selected RNA was eluted by boiling for 1 min in 30 μl of H_2O containing 5 μg of calf liver tRNA, followed by quick freezing in a ethanol/dry ice bath. The supernatant was made 0.2 M in NaOAc and precipitated with 3 vol of ethanol.

RESULTS

Induction of Nitrate Reductase by Nitrate. Barley seedlings were labeled *in vivo* with [^{35}S]methionine with or without nitrate induction, and the crude protein extracts were immunoprecipitated with a nitrate reductase-specific polyclonal antiserum (7). Examination of these immunoprecipitates by $NaDodSO_4$/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) showed a dramatic increase in the 110-kDa nitrate reductase protein band in induced compared to uninduced (control) material (Fig. 1*a*, lanes 1 and 2, respectively). RNA extracted from similarly treated seedlings was translated *in vitro* in a reticulocyte cell-free system and the products were immunoprecipitated. A 110-kDa immunoprecipitable band, which comigrated with the *in vivo* labeled nitrate reductase, was markedly induced in the nitrate-grown plants (Fig. 1*b*, compare lanes 1 and 2). Thus, the induction of nitrate reductase protein correlated with an increase in translatable nitrate reductase mRNA.

Isolation of cDNA Clones for Nitrate Reductase mRNA. To further study the basis of this induction, a cDNA clone for nitrate reductase was isolated. Based on *in vitro* translation and immunoprecipitation results, the relative abundance of induced nitrate reductase mRNA in the total poly(A)$^+$ RNA was estimated to be about 0.01%. The size of the nitrate reductase polypeptide, 110 kDa (native nitrate reductase is a 220-kDa homodimer) (21), requires a minimum mRNA size of 3 kilobases (kb). The relatively low abundance and large size of nitrate reductase mRNA suggested enrichment by size fractionation prior to cDNA synthesis. Total poly(A)$^+$ RNA from nitrate-induced barley leaves was fractionated on a native sucrose gradient (data not shown). The fractions containing the nitrate reductase mRNA were identified by *in vitro* translation, followed by immunoprecipitation. This population of poly(A)$^+$ RNA, enriched 5- to 10-fold for nitrate reductase mRNA, was used to construct a cDNA library in the expression vector λgt11 (17). Twenty-five thousand recombinants were screened with the nitrate reductase antibody (7, 17). Two independent clones (bNRλ1 and bNRλ2) reacted with nitrate reductase antibody. The cDNA inserts in these clones cross-hybridized and restriction mapping suggests that they represent overlapping clones from a single transcript (data not shown).

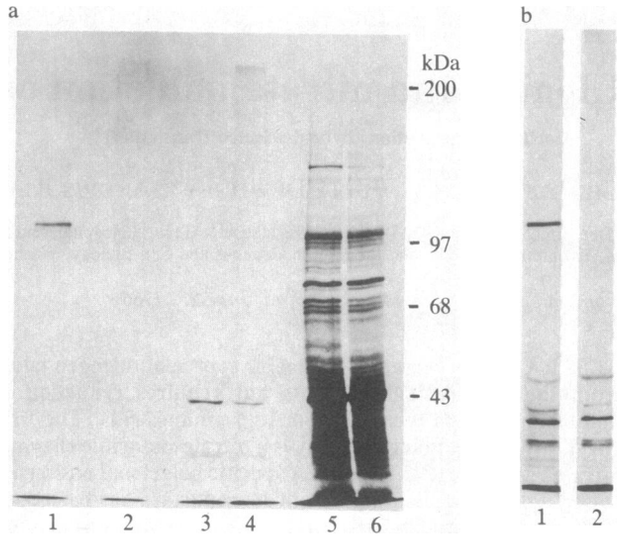

FIG. 1. Induction of nitrate reductase by nitrate. (*a*) Immunoprecipitation of *in vivo* [^{35}S]methionine-labeled protein. Total labeled proteins extracted from plants treated with medium plus nitrate (induced, lane 5) and minus nitrate (uninduced, lane 6) were separated on a $NaDodSO_4$/polyacrylamide gel and the gel was radioautographed. Aliquots of the same total labeled proteins shown in lanes 5 and 6 were immunoprecipitated with a nitrate reductase specific polyclonal antibody (4) (lane 1, induced; lane 2, uninduced) or with preimmune serum (lane 3, induced; lane 4, uninduced). (*b*) Immunoprecipitation of *in vitro* [^{35}S]methionine-labeled protein. Two hundred nanograms of poly(A)$^+$ RNA isolated from plants treated with medium plus nitrate (induced, lane 1) and minus nitrate (uninduced, lane 2) was translated *in vitro* and the products were immunoprecipitated with nitrate reductase antibody. Electrophoresis was in a 7.5% $NaDodSO_4$/polyacrylamide gel. The positions of size standards (kDa) are shown from top to bottom: myosin (H-chain), 200 kDa; phosphorylase B, 97 kDa; bovine serum albumin, 68 kDa; and ovalbumin, 43 kDa.

Another cDNA library was made with mRNA highly enriched for nitrate reductase mRNA by the following procedures. The poly(A)$^+$ RNA population, enriched for nitrate reductase mRNA by sedimentation in a sucrose gradient, was further enriched by methylmercuric hydroxide/agarose gel electrophoresis (Fig. 2) (18). The location of the nitrate reductase mRNA in the gel was determined by *in vitro* translation of the mRNA extracted from gel slices and immunoprecipitation of the synthesized nitrate reductase protein. One hundred to 200-fold enrichment was achieved by this two-step fractionation, resulting in a population containing about 1–2% nitrate reductase mRNA. Gel slice no. 5 (Fig. 2) contained ≈100 ng of RNA, of which ≈1.6% was nitrate reductase mRNA (estimated by *in vitro* translation and immunoprecipitation). Using this 100 ng of RNA, a (dG-dC)-tailed cDNA library of 4000 transformants was constructed in the plasmid vector pUC12 (19). Initial screening of 1000 clones with radiolabeled insert DNA from bNRλ1 and bNRλ2 gave 10 positively hybridizing clones, a number consistent with the estimated level of nitrate reductase mRNA in the enriched population. The longest clone among these 10 clones, bNRp10, contains a 1.1-kb cDNA insert.

Hybrid Selection of Nitrate Reductase mRNA with bNRp10. Hybrid-selection translation was used to confirm the identification of bNRp10 as a nitrate reductase cDNA clone. Total poly(A)$^+$ RNA extracted from induced barley seedlings was hybridized with either bNRp10 or pUC12 DNA, each immobilized on separate nitrocellulose filters; RNA recovered from each filter was then translated *in vitro*. Translation of bNRp10-selected mRNA yielded a single 110-kDa product on

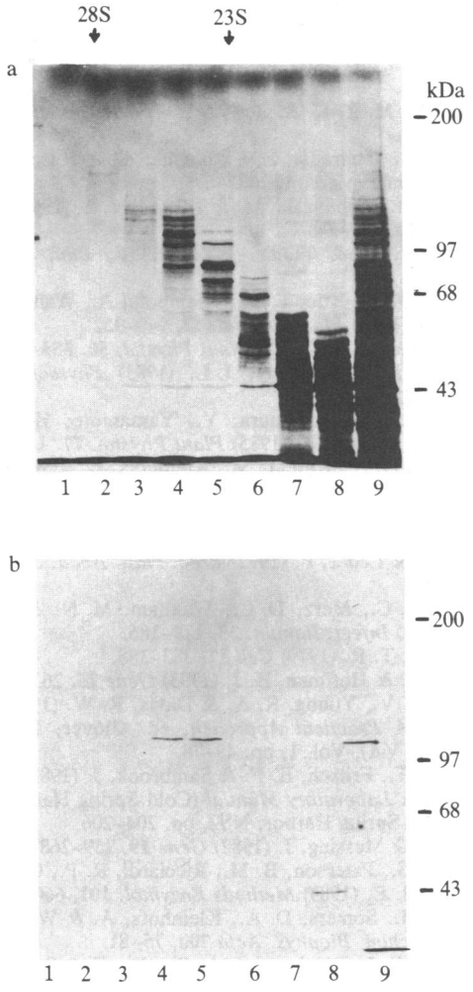

FIG. 2. Fractionation of mRNA by methylmercuric hydroxide/agarose gel electrophoresis followed by *in vitro* translation (*a*) or *in vitro* translation and immunoprecipitation with nitrate reductase antibody (*b*) and separation of the products in both cases by $NaDodSO_4$/PAGE. Each lane in *a* shows the translation products of the RNA eluted from a single gel slice and each lane in *b* shows the immunoprecipitation of the same products. Lane 9 displays the translation products of total poly(A)$^+$ RNA. Size markers and the conditions used for $NaDodSO_4$/PAGE are the same as in Fig. 1. The positions of the rRNA markers were determined after electrophoresis in an adjacent lane on the methylmercuric hydroxide/agarose gel and were visualized by ethidium bromide staining.

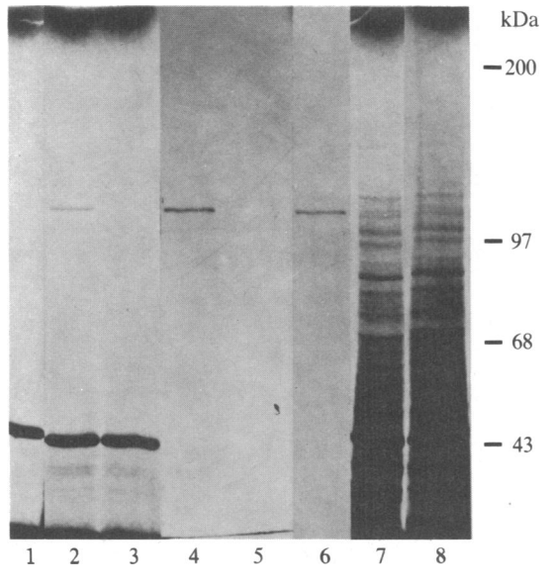

FIG. 3. Hybrid selection with bNRp10 DNA followed by *in vitro* translation. Poly(A)$^+$ from nitrate-induced plants was hybridized with filter-bound bNRp10 or pUC12 DNA. RNA was then eluted from the filter and *in vitro* translated, and the translation products were separated by $NaDodSO_4$/PAGE. Lanes 1–3, *in vitro* translation with no added RNA (lane 1), with RNA hybrid selected by bNRp10 (lane 2), and with RNA selected by pUC12 (lane 3); lanes 4 and 5, immunoprecipitation products of lanes 2 and 3, respectively; lane 6, immunoprecipitation product of *in vitro* translated total poly(A)$^+$ RNA whose translation products are shown in lane 7; lane 8, *in vitro* translation products of RNA left in solution after hybrid selection with bNRp10 (i.e., the unhybridized RNA from the reaction shown in lane 2). Size markers and $NaDodSO_4$/PAGE conditions were as in Fig. 1.

$NaDodSO_4$/PAGE (Fig. 3, lane 2), which was not seen when pUC12 DNA-selected mRNA was translated (Fig. 3, lane 3). Furthermore, the nitrate reductase antiserum immunoprecipitated the 110-kDa translation product (Fig. 3, lane 4), which, in turn, comigrated with the immunoprecipitation product of translated total poly(A)$^+$ RNA (Fig. 3, lane 6).

Induction of Nitrate Reductase mRNA by Nitrate. A RNA transfer blot, in which equal amounts of poly(A)$^+$ RNA from nitrate-induced (Fig. 4*a*, lane 1) and uninduced control (Fig. 4*a*, lane 2) barley plants were hybridized with a bNRp10 nick-translated probe, showed a dramatic induction of a single 3.5-kb transcript. The size of the hybridizing RNA (3.5 kb) agrees with the apparent size of the RNA that translated into immunoprecipitable nitrate reductase observed in a methylmercuric hydroxide/agarose gel (Fig. 2*b*). The same filter used in Fig. 4*a* was washed to remove the bound radioactive bNRp10 DNA probe and hybridized to a control cDNA (2E7) isolated from the plasmid library. The insert of clone 2E7 corresponded to a relatively abundant transcript that encodes a 20-kDa peptide (data not shown). Clone 2E7, as well as the majority of the cDNA clones, showed no preferential hybridization to reverse-transcribed cDNA probes made with either induced or noninduced mRNA (data not shown). Therefore, the equal intensity of the 1-kb bands in Fig. 4*b* demonstrated that equal amounts of intact RNA were present in the two lanes on the RNA transfer blot.

DISCUSSION

Although the induction of nitrate reductase by its substrate was reported in 1957 (2), the mechanism of induction is not known. We used a specific polyclonal antibody for barley nitrate reductase to precipitate *in vitro* translated mRNA; a marked increase of translatable nitrate reductase mRNA upon nitrate induction of the plants was shown. A λgt11 library was screened using this antibody and two short cDNA clones, bNRλ1 (≈350 bp) and bNRλ2 (≈500 bp), were obtained. We then used *in vitro* translation and immunoprecipitation as an assay to enrich nitrate reductase mRNA ≈200-fold by a two-step size fractionation procedure. The cDNA library constructed with this RNA in pUC12, originally intended for direct hybrid-selected screening, was screened with insert DNA from bNRλ2 as probe in order to obtain clones with longer cDNA inserts (e.g., bNRp10). With the cDNA to nitrate reductase mRNA cloned, we were able to show the increase of steady-state mRNA level in response to nitrate induction of the plants. Data are not yet available to determine whether this increase is due to changes in transcriptional rate, altered RNA processing, or decreased mRNA degradation.

Due to the important role of nitrate reductase in nitrogen assimilation, a large body of information concerning the enzyme and its regulation is available (1, 24, 25). Also, due

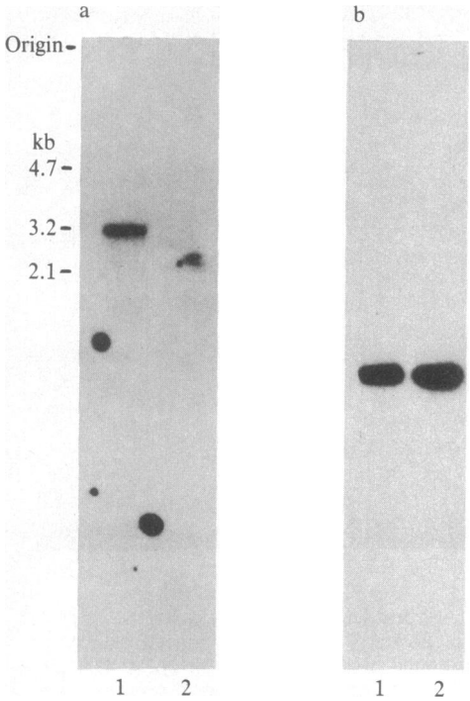

FIG. 4. RNA blot-hybridization analysis of nitrate reductase mRNA. (*a*) The nitrocellulose filter was hybridized to nick-translated (22) insert DNA from bNRp10 (10^6 cpm/ml; 2×10^8 cpm/μg). (*b*) The filter was hybridized to nick-translated insert DNA [≈700 base pairs (bp)] from 2E7 (10^5 cpm/ml; 2×10^8 cpm/μg). Each lane contains 10 μg of poly(A)$^+$ RNA from plants treated with medium plus nitrate (lane 1) and minus nitrate (lane 2). RNA was denatured in glyoxal and dimethyl sulfoxide, electrophoresed in a 1% agarose gel, and transferred to a nitrocellulose filter as described (23).

to the ease of chlorate selection schemes for nitrate nonutilizing mutants, whole plant and cell line mutants deficient in nitrate reductase are available for many species of plants (26–29). We have demonstrated in this manuscript that nitrate induction increases the steady-state level of nitrate reductase mRNA. With a cDNA for nitrate reductase cloned, it should now be possible to analyze at the molecular level nitrate reductase gene regulation both biochemically and genetically.

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